Alternate Activation of Microglia as a Protective Adaptation against Chronic Neuroinflammation in Young Rats

Undergraduate Honors Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Neuroscience in the Arts and Sciences college of The Ohio State University

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Abstract

Alzheimer's disease (AD) and other age related neurodegenerative diseases share a chronic neuroinflammation component in different areas of the brain. The sustained presence of neuroinflammation is harmful due to the prolonged release of toxic cytokines by activated microglia, which results in damage to the body's own cells. To model chronic neuroinflammation, a cannula delivered lipopolysaccharide (LPS) into the fourth ventricle of three month old rats for periods of two, four, and eight weeks. Following two and four weeks infusion, the rats showed impaired spatial memory performance on the Morris Water Maze task compared to the control group receiving artificial cerebrospinal fluid. However, after eight weeks of LPS administration, young rats exhibited a compensatory adaptation, which returned spatial memory performance to that of the control group. It was hypothesized that the microglia in the young brain had shifted from the classical M1 phase associated with damaging defense to the alternative M2a activation associated with repair. The correlation of Morris Water Maze performance and immunohistochemical analysis of Major Histocompatability Complex II and Peroxisome Proliferator-Activated Receptor gamma expression failed to support this hypothesis. Further understanding the underlying less destructive path will provide possible targets for intervention in AD, as inducing these adaptations may prevent further damage.

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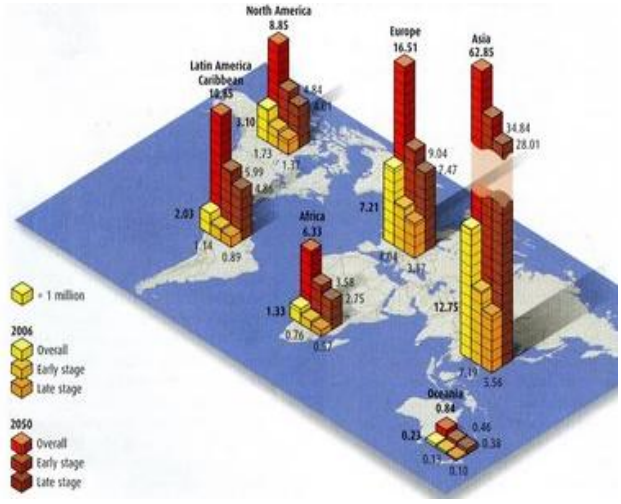


Figure 1. Worldwide Prevalence of Alzheimer's disease. Using data from the United Nations worldwide population forecasts, it was projected that with the aging population, the worldwide prevalence of AD is expected to rise to 106.2 million by the year 2050 (Brookmeyer *et* al., 2007).

In 2006, 26.6 million people world-wide suffered from Alzheimer's disease (AD), and the prevalence is estimated to quadruple by the year 2050, affecting 1 in 85 people (Figure 1) (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007). AD is a debilitating neurodegenerative disorder that primarily affects the aged population. The principle behavioral symptom which manifests in AD patients is memory impairment. Someone with AD may get lost in once familiar places, forget biographical information, and become disoriented to time and place (Förstl & Kurz, 1999). This behavioral deficit is linked to the extensive atrophy of the hippocampal region, which is associated with learning and memory (reviewed in Gu, Jiang, & Huang, 2010).

While the molecular basis of AD pathology remains elusive, researchers have begun to uncover its complex networks. Research indicates that both aggregates of beta-amyloid (Aβ) and tau proteins initiate a cascade of immune system responses that includes the formation of chemokines, cytokines, and membrane attack complexes (Figure 2) (Shen *et al.*, 2001). Though hallmark clinical features of inflammation, such as swelling, heat, redness, and pain, do not manifest in AD, evidence of neuroinflammation is prominent in the tissue of those affected by this neurodegenerative disorder (reviewed in Gasparini, Ongini & Wenk, 2004). Current AD

research aims to connect the mechanisms of neuroinflammation with the etiology and treatment of AD.

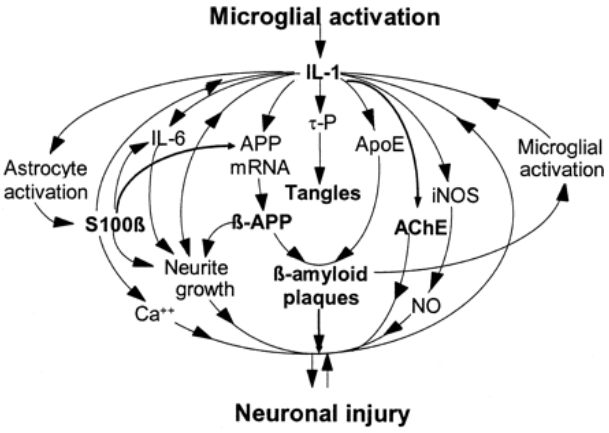


Figure 2. Pathological cycle in Alzheimer's Disease. The neuropathologic features of Alzheimer disease include ß-amyloid plaques, neurofibrillary tangles, synapse and neuronal dysfunction and loss, reduced neurotransmitter concentrations, and activation of glial cells with expression of proinflammatory cytokines (Sue & Griffin, 2006).

A healthy individual has minimal baseline inflammation. Following injury or pathogenic invasion, the body can mount both selective and non-selective immune responses. Upon sensing small chemical changes in the environment, cells called macrophages become activated and travel to the site of damage, where they engulf pathogens and debris; microglia are specialized macrophages found in the central nervous system (CNS) (reviewed in Kreutzberg, 1995). Inside the macrophage, Major Histocompatibility Complex II (MHCII) picks up a piece of partially digested protein and presents it outside the cell to attract a specific T cell (Figure 3). For the non-specific response, highly reactive molecules, including nitric oxide (NO) and reactive oxygen species (ROS), are released from the macrophage and cause indiscriminative damage to surrounding material. In addition, inflammatory mediators called cytokines are released, including

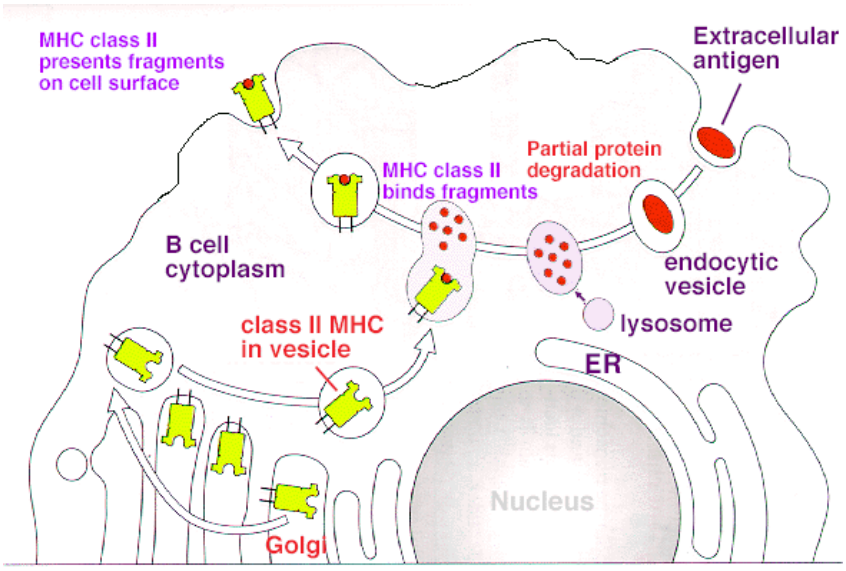


Figure 3. The role of MHC II. MHC class II displays partially digested antigens on the surface of activated microglia to attract appropriate T cells of the immune system. (Parham, 2000)

interleukin(IL)-6, tumor necrosis factor (TNF)-α, and IL-1β (reviewed in Miyata & Eeden, 2011). Acute activation of this immune cascade is beneficial by destroying invaders and cleaning up cellular debris. However, chronic activation of this cascade inflicts considerable damage to cells that the body relies on for proper functioning of memory, affect, and cognition. It is thought that the "balance" of these pro- and anti-inflammatory signals may determine the susceptibility and outcome of disease (reviewed in Rubio-Perez & Morillas-Ruiz, 2011).

The typical aged brain contains an overall increased level of inflammation, which proves to have damaging effects on learning and memory. An increase in the pro-inflammatory cytokine IL-1β leads to decreased neurogenesis, an essential component to hippocampal function and memory formation (Kuzumaki *et al.*, 2010). This age-related increase of IL-1β is accompanied by a decrease in the anti-inflammatory IL-4 cytokine in the hippocampus of aged rats with poor Long Term Potentiation (LTP) (Maher, Nolan, & Lynch, 2005). In a transgenic mouse model of AD, increased production of the proinflammatory cytokine IL-6 leads to inflammatory neurodegeneration and impairs memory task performance, including avoidance learning (Heyser, 1997). IL-6 also compromises hippocampal neurogenesis (Vallieres, Campbell, Gage, & Sawchenko, 2002). Restoring the balance of pro-inflammatory and anti-inflammatory factors could potentially prevent further hippocampal damage and return memory function towards baseline (Figure 4).

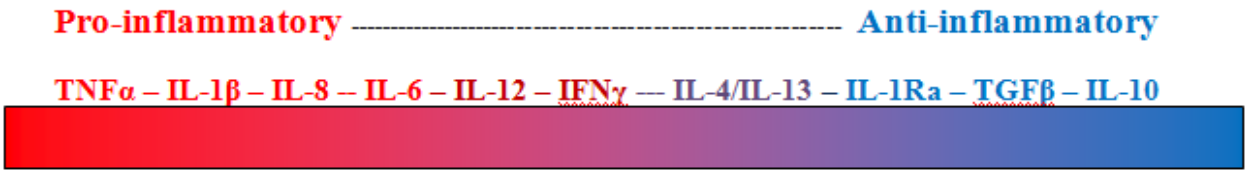


Figure 4. Spectrum of Cytokines Regulating Inflammation. Cytokines are chemicals that regulate the level of inflammation. While each cytokine generally has its own broad spectrum of actions, some are thought it be more potent in their actions than others. (reviewed in Petermann & Korn, 2011). There exists a general trend from extremely pro-inflammatory to extremely anti-inflammatory. The balance among them determines the overall amount of inflammation.

In the rodent model of chronic neuroinflammation, chronic administration of lipopolysaccharide (LPS) into the fourth ventricle reproduces many of the behavioral, neurochemical, and neuropathological changes associated with AD (Hauss-Wegryzyniak, Dobrzanski, Stoehr, & Wenk, 1998). It provokes extensive inflammatory responses in the hippocampus and its subregions, including the increase of IL-1β and TNFα (Hauss-Wegryzyniak *et al*., 1998). Microglia from aged brains have a more intense reaction to LPS than microglia in younger brains; this results primarily in greater increases in glial expression of IL-6 and IL-1 (Xie, Morgan, Rozovsky, & Finch, 2003). Studies with transgenic mice lacking the IL-6 gene demonstrate that without IL-6, high circulating levels of TNFα and IL-1β are not sufficient to disrupt working memory (Sparkman *et al.*, 2006). This suggests that the increased IL-6 in aged brains may exacerbate the cognitive decline associated with inflammation.

In addition to the damaging, defensive role, microglia can also specialize in the resolution of inflammation and the repair process (Figure 5) (Mills, Kincaid, Alt, Heilman, & Hill, 2000). The "classical" (M1) macrophage is associated with the oxidative damage seen in AD and releases the pro-inflammatory cytokines TNFα, IL-1, and IL-6 (Khoury *et al.*, 2007). Increased

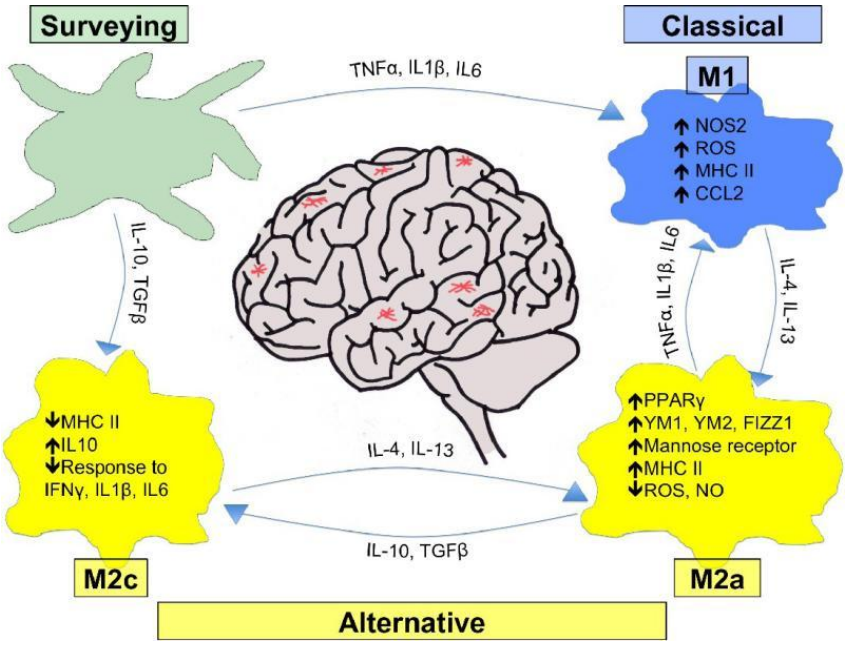


Figure 5. Markers for macrophage phenotypes. There are multiple phenotypes of activated microglia. Increased MHC II is a marker common to both M1 and M2a activation. The present experiment aims to use PPARγ as a marker unique to M2a (Cameron & Landreth, 2010).

MHCII has been used as a marker for M1 activation. Negative feedback mechanisms trigger the reduction of the classical pro-inflammatory response (reviewed in Colton, 2009). The transition that decreases the inflammatory response and supports tissue repair includes the release of a number of anti-inflammatory cytokines, including IL-4 and IL-13 (reviewed in Colton, 2009). In the presence of IL-4 and IL-13, macrophages may transition into a true anti-inflammatory state of activation (Stein, Keshav, Harris, & Gordon, 1992). This repair state of macrophages has been coined "alternative activation" (M2a) and is characterized by the release of anti-inflammatory cytokines IL-10 and IL-1RA (IL-1 receptor antagonist) (Rutschman et al., 2001). Further, M2a uses the enzyme arginase to convert the amino acid L-arginine into urea and ornithine, instead of the destructive NO (Rutschman et al., 2001). MHCII is also displayed in alternative activation, but unique markers include PPARγ, YM1, YM2, and FIZZ1 (reviewed in Cameron & Landreth, 2010). It remains to be determined if and when this phenotypic switch is induced in the presence of chronic neuroinflammation.

Using a transgenic mouse model of AD, Jimenez et al. (2008) observed an age-dependent switch in microglial activation between M1 and M2a. The expression of inflammatory markers TNFα and iNOS were only slightly altered in 6-month-old mice compared to wild type mice. In 12-month-old transgenic mice, there was a moderate increase in TNFα expression. By the age of 18 months, the expression of both TNFα and iNOS had significantly increased in the hippocampus of the transgenic mice. This suggests a harmful switch from the alternative to the classic microglial activation is restricted to old ages and may account for the cognitive decline seen in AD (Jimenez et al., 2008).

To reproduce the chronic inflammatory component of AD and study its consequences, the LPS model is used. Previous work has shown that young (3 m.o.) animals which undergo

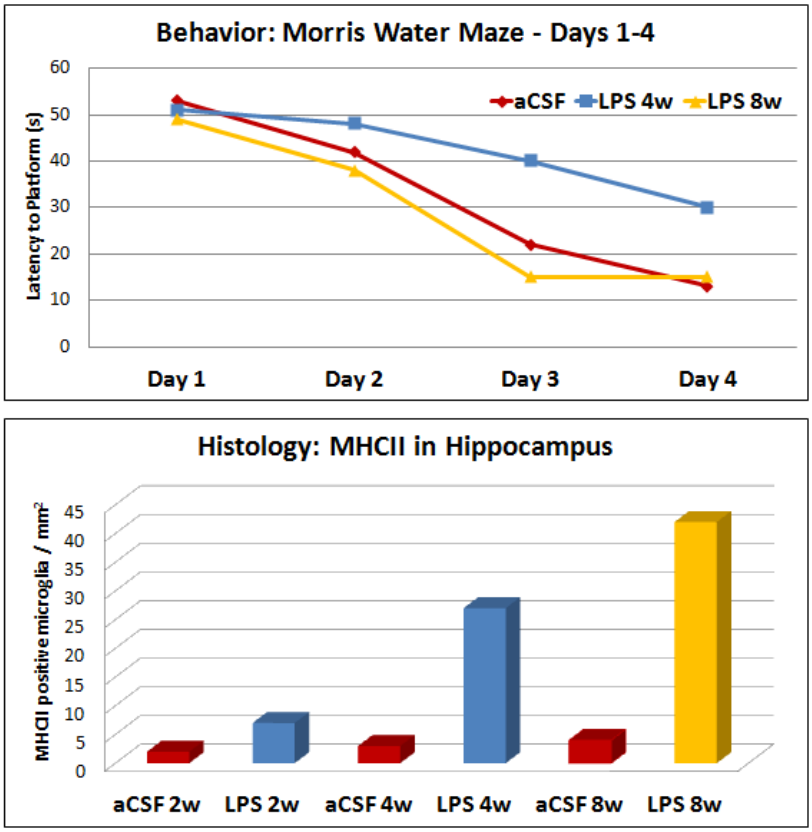


Figure 6 (Above) Morris Water Maze Results: Through the first four weeks, LPS infusion leads to a decrease in MWM performance. By the 8th week, a compensatory mechanism arises to improve spatial memory in young rats.

(Below). Major Histocompatability Complex II Immunohistochemistry Densitometry. MHCII is a marker for activated microglia. As duration of LPS infusion increases, the number of activated microglia increases (Brothers, unpublished data).

LPS infusion over periods of two and four weeks mimic the increased neuroinflammation and poor spatial memory performance in the Morris Water Maze (MWM) seen in untreated aged (23 m.o.) animals (Brothers, Marchalant, & Wenk, 2010; Marchalant, Cerbai, Brothers, & Wenk, 2008). A prior experiment by Brothers (2010) induced chronic neuroinflammation in young rats (3 m.o.) via LPS, and it was expected that the performance on the MWM would be inversely proportional to the amount of MHCII staining; increased damage from more activated microglia would lead to poorer performance. Instead, the results showed that this relationship only holds true through the first four weeks. By the eighth week, it appears that a compensatory mechanism arises to improve spatial memory performance in young rats, while MHCII expression levels continue to rise (Figure 6) (Brothers, unpublished data).

The present study aims to determine if a change in microglial activation is the phenotypic switch seen in young brains. The hypothesis states that following chronic neuroinflammation,

microglial activation changes from the M1 "classical activation" to the M2a "alternate activation" state to minimize neuronal damage. Therefore, while the increased MHCII shared by both states continues to increase throughout the eight weeks post surgery, the PPARγ that is characteristic of the alternate state is expected to increase between weeks four and eight (Figure 7). If these adaptive mechanisms of young brains are better understood, they may be able to be induced in aged brains to protect against the ill effects of chronic neuroinflammation.

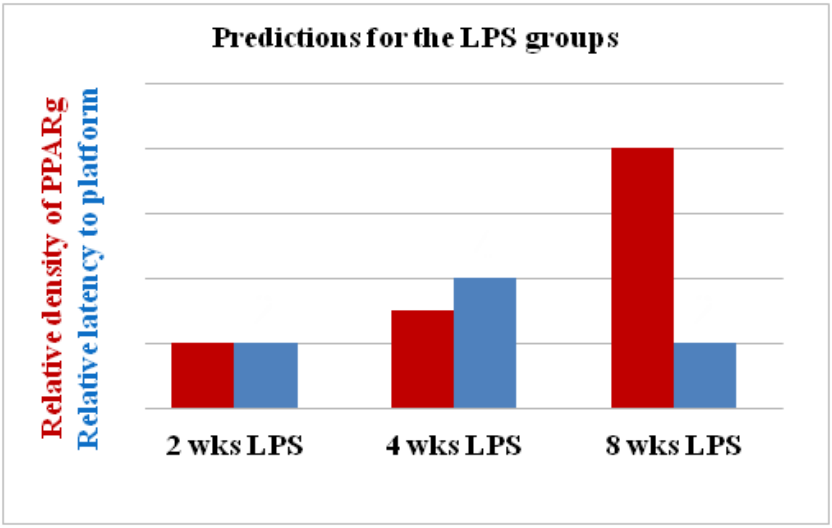


Figure 7. Predicted Results. The prediction states over time the relative density of PPARγ will increase and the latency to reach the platform will decrease.

Methods

As previously described (Hauss-Wegrzyniak et al., 1998), sixty young male Fischer rats underwent surgical implantation of an osmotic mini-pump which delivered a chronic infusion of lipopolysaccharide (LPS) or artificial cerebral spinal fluid (aCSF) via a cannula into the IVth ventricle; two, four, or eight weeks later, the animals underwent behavioral testing with the Morris Water Maze. Following sacrifice, immunohistochemistry was utilized to examine PPARg protein expression.

Subjects and Surgery

Sixty young (4 m.o.) male Fischer rats (Harlan Sprague–Dawley, Indianapolis, IN) were individually housed in Plexiglas cages with free access to food and water. The rats were maintained on a 12-h light/dark cycle in a temperature-controlled room (22˚C) with lights off at 09:00. Health of the rats was assessed upon arrival and throughout the experiment. During the first week, they were familiarized with their new environment and handled by the investigators prior to surgery.

Cannula Implantation

Each rat was anesthetized with isoflurane and securely placed into a stereotaxic apparatus. After making an incision, a hole was drilled down to dura (at approximately -2.5AP, 0.0LM). A cannula was inserted into the fourth ventricle and secured with acrylic. A subcutaneous osmotic minipump (Alzet model #2006, to deliver 0.15µl/hr; Durect Corp., Cupertino, CA, USA) and tubing (Tygon tubing, 0.06 O.D.) were implanted to deliver the reservoir of fluid to the cannula. This allowed aCSF (140 mM NaCl, 3.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 1.2 mM Na_2HPO_4 adjusted to pH 7.4) or LPS (0.25µg/hr, 1.66

mg/ml dissolved into aCSF; Sigma, St. Louis, MO, USA E. coli, serotype 055:B5, TCA extraction) to be chronically infused for a period of up to eight weeks. Animals were sutured, given lidocane, and fed a high caloric diet until they appeared to be gaining weight normally.

Behavioral Testing

The Morris Water Maze (MWM) was used to assess spatial learning ability. The animals were tested two, four, or eight weeks following surgery. A clear, circular platform (10 cm in diameter) was submerged 2.5 cm underwater inside a 185 cm diameter pool and kept in a constant location. The testing site had white walls with proximal and distal visual clues. The rats were tracked by an overhead video camera connected to a tracking unit (Nagedus EthoVision 3.1, Nagedus, Leesburg, VA), which recorded latency to find the platform, distance traveled, speed, and other variables.

On the first day, rats were placed upon the hidden escape platform for 30s. Subsequently, they completed three sets of two consecutive trials (6 trials/day) with 1 hour between sets, and repeated this over the next three days (24 trials total). During each trial, the animal was randomly placed into the pool from one of six entry points that were evenly spaced along the edge of the pool. The trial ended after the rat found the hidden platform or 60s had elapsed. The animal remained or was placed on the platform for an additional 30s following each trial. Latency to find the platform across days was analyzed. Measures of general movement such as swim speed and thigmotaxis were also analyzed for possible motor impairments. On the fifth day, four regular trials were run and recorded. One probe trial (without platform) was performed to ensure that the rat cannot see the submerged platform; good test performance showed the rat spending

the most time in the quadrant that formerly housed the platform. Two visible platform trials (2 cm above water) were performed to ensure the rat was free of visual deficits.

Histological Procedures

Fixation

Following the behavioral testing, each rat was anesthetized with isoflurane and underwent a transcardiac perfusion (10ml/min) with saline (0.9%, 80mL) containing heparin (1 U/mL), followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4, 120 mL). Brains were removed and stored in the 4% paraformaldehyde fixative solution at 4˚C, then transferred to PBS. The brains were sliced into 40μm coronal sections via vibratome (Leica) and stored in an antifreeze solution (0.5 M phosphate buffer with glycol ethylene and glycerol) until staining.

Immunohistochemistry

Slices containing the hippocampus and the cingulate cortex were selected for immunohistochemistry to visualize the transcription factor PPARgamma. Unless otherwise noted, all steps were performed on an agitator at 22˚C; all rinses were performed with PBS (1M 3x10min). The tissues were rinsed to clear antifreeze, then mounted onto slides and outlined with a hydrophobic pen (Liquid Blocker).

Antigen Retrieval

The slides were put into boiling 0.1 M citric acid (pH 6.0; microwave 2 min, power 10) and microwaved (5 min, power 2), then allowed to cool at rt. A trypsin digest (abcam, #ab970)

was performed for 15 min and rinsed. This aimed to enhance the signal of immunohistological staining.

Peroxidase Reaction Staining

A quench was performed with 0.3% H_2O_2, 10 M PBS, and quenching stock for 60 min to block endogenous peroxidase activity and rinsed. Blocking was performed in NGS (5%) to prevent non-specific staining. The tissues were incubated in a PPARγ primary antibody (1:100 dilution, rabbit polyclonal, Santa Cruz, #SC-7196) at 4˚C overnight. The following day, the tissue was rinsed then bathed in the biotinylated goat-antirabbit 2˚ antibody (1:200 dilution, 120min, Vector, Burlingame, CA, #BA-1000) for 120 min. After rinsing, the tissue was treated with an avidin-biotinylated horseradish peroxidase Elite Kit (Vectastain, Elite ABC kit, Vector), then rinsed again. The tissue was stained using 3,3'-diaminobenzidine tetrahydrochloride as chromogen (2.5mL PBS, 125µL 1% H_2O_2, 125µL 1% DAB, Vector, Burlingame, CA) until desired color was reached (5 min), and the reaction was stopped by rinsing with buffer. The tissue was rinsed in 1M PBS and 95% ethyl alcohol. The slides were dehydrated (2 min each of 75% EtOH, 90% EtOH, 100% EtOH, 100%EtOH, xylene, xylene) and coverslipped with cytoseal mounting medium.

Histological Analysis

The tissues were examined using brightfield light microscopy (Nikon 90i microscope and NIS Elements software). Histochemical examination of the hippocampus was performed using two representative slides per animal.

Results and Discussion

Observations from immunohistochemistry fail to support the hypothesis that young brains transition to a less destructive form of microglial activation under conditions of chronic neuroinflammation. There were no distinctive differences in the patterns of PPARγ staining acquired by the tissue between experimental groups over time (Figure 8). Previous OX-6 staining

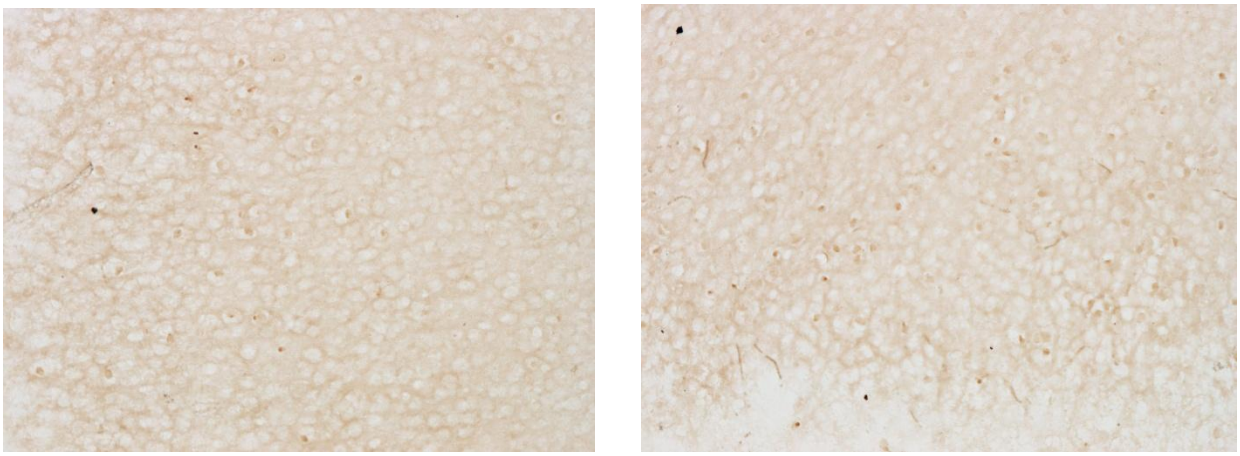
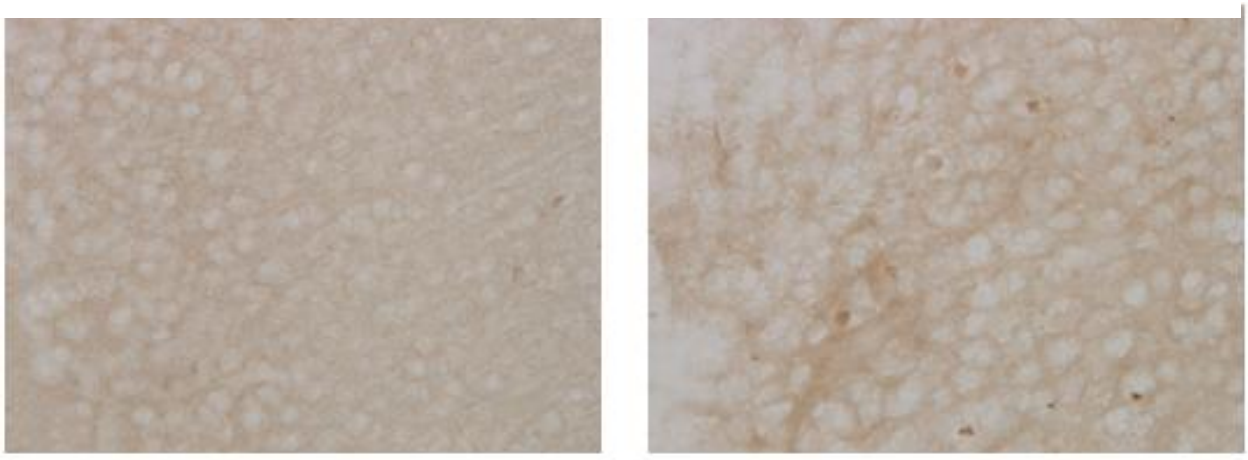


Figure 8. PPARγ Immunohistochemistry. There were no distinctive differences in the patterns of PPARγ staining acquired by the tissue between experimental groups over time, including LPS 4 weeks (10x, sc 1:100, upper left; 20x, sc 1:100 bottom left) and LPS 8 weeks (10x, sc 1:100, upper right; 20x, sc 1:100, bottom right. However, a considerable amount of non-specific background staining was present.



in our lab has demonstrated that the young animals increase the amount of MHC-II synthesized as the duration of LPS infusion increases (Brothers, unpublished data). This MHC-II can be a marker for either M1 activation or M2a activation (reviewed in Cameron & Landreth, 2010). The transcription factor PPARγ was chosen as a marker because high expression levels of it are a

characteristic of M2a microglial activation, but not M1 activation (reviewed in Cameron & Landreth, 2010). The coloration in the PPARγ stain was diffuse throughout the brain and appeared to be primarily localized to intercellular space, but the PPARγ transcription factor should be localized to the nucleus. A review by Bordeaux et al. examines the high frequency of non-specific antibodies on the market and increases confidence in this claim of poor antigenic accuracy (2010). Without a reliably measured comparison in PPARγ between groups, no conclusion can be drawn about the activation state of the microglia. There may be no change in activation state or current PPARγ antibodies may be inadequate.

After following the protocol from two different manufacturers (abcam, ab-19481; santa cruz; sc-7196), the resulting stains did not resemble the sample photos they provided (Figure 9). Much of the color acquired by the tissue was thought to be non-specific background staining. Various measures were taken to increase the permeability of the membranes to reach the intranuclear PPARγ protein and enhance the signal of the immunohistological stain. Tween or TritonX-100 was added to the phosphate buffered saline solution to act as a detergent. Heat Induced Epitope Retrieval was performed using Tris-EDTA and citrate buffers in waterbaths and microwaves of varying durations and temperatures. Proteolytic Induced Antigen Retrieval was performed with a trypsin digest. Individual and combinations of these pretreatments only provided minor improvements, if any. Because PPARγ is known to be an intranuclear protein, one measure of the specificity of the antibody would examine its

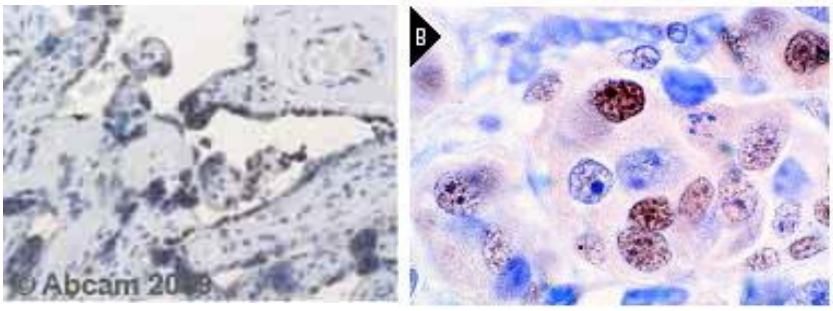


Figure 9. Abcam and Santa Cruz sample PPARγ IHC images. The above images were representative IHC stains shown from two primary antibody vendors, Abcam (left) and Santa Cruz (right).

localization. Eosin B, a cytoplasmic stain, was used in combination with PPARγ and no clear localization of the PPARγ protein was found. The poor results of the immunohistochemical stain, despite many protocols borrowed from other successful PPARγ researchers, suggests that current PPARγ antibodies are ineffective for the examination of brain tissue.

Despite the lack of success with current polyclonal antibodies, the possibility of microglial activation mediating a repair state remains an important mechanism to be examined. If clear results cannot be reached using PPARγ immunohistochemistry, then other avenues must be explored. One possible direction is to measure other markers that are also unique to the M2a state, such as the levels of expression of FIZZ1, YM1, YM2, and mannose receptors (reviewed in Cameron & Landreth, 2010); perhaps these other markers have more effective antibodies available. Another way to approach the research question would be to directly monitor the cytokine profile expressed following chronic neuroinflammation. A predominant presence of TNFα, IL-1β, and IL-6 would suggest the M1 activation, whereas a change to IL-10 and IL-1Ra would suggest the M2a activation (Khoury *et al.*, 2007; Rutschman et al., 2001). Many avenues are available to continue the study of the microglial response to chronic neuroinflammation (Figure 10).

Macrophage Phenotype	M1 – Classical	M2a - Alternate
Activating signals	LPS, IFNγ, TNF	IL-4, IL-13, glucocorticoids
Secretory products (direct or indirect)	↑TNFα, IL-1β, IL-6, ↑IL-12	IL-10, IL-1Ra
Biological markers	↑MHC II, ↑CD86, ↓Mannose receptor	↑Mannose receptor, ↑Scavenger receptor, ↑CD23, ↓CD14, CD163, MS-1
Killer molecules	NO, O_2^-	None

Figure 10. Macrophage Functional Phenotypes. Each state of microglial activation has characteristic activating signals, secretory products, and biological markers.

It is important to continue to extract information from the LPS paradigm in order to examine the immune response of the brain following the periods of chronic neuroinflammation experienced with many neurodegenerative diseases affecting the aged. Following mild brain injury, the young human brain exhibits greater long-term cognitive functioning compared to the aged brain (Senathi-Raja, D., Ponsford, J., & Schönberger, M., 2010). This pattern is effectively mirrored by our LPS paradigm. By the eighth week of a chronic immune response, the spatial memory of the young experimental LPS group recovers to that of the young control group (Brothers, unpublished data). However, old (24 mo) rats do not show this return to baseline (Marchalant et al., 2008). Further understanding the underlying less destructive response exhibited by young brains will provide possible targets for intervention in AD, as inducing these adaptations may prevent further damage.

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